Human Papillomavirus Type 16 DNA from a Vulvar Carcinoma in situ is Present as Head-to-Tail Dimeric Episomes with a Deletion in the Non-coding Region

By IAIN M. KENNEDY,* STUART SIMPSON, JOAN C. M. MACNAB AND J. BARKLIE CLEMENTS

Institute of Virology, University of Glasgow, Glasgow G11 5JR, U.K.

(Accepted 6 October 1986)

SUMMARY

A number of genital cancer biopsy samples were screened for the presence of human papillomavirus type 16 (HPV-16) DNA sequences. One of these samples (a vulvar carcinoma in situ) was found to contain more than 100 copies of HPV-16 DNA sequences per cell. Using this tumour DNA, a genomic library was constructed in bacteriophage lambda and the library was screened for recombinant phage containing HPV-16 sequences. Five recombinant phage clones were isolated and their DNA was analysed by restriction endonuclease digestion and blot hybridization. All five recombinants contained two copies of the HPV-16 genome present in a head-to-tail arrangement. The data are consistent with the presence of HPV-16 sequences in the tumour DNA arranged as genomic dimers in a circular episomal configuration. The HPV-16 genomes contained a deletion within the non-coding region, a region which includes the viral origin of DNA replication and transcriptional control sequences. Possible consequences of this deletion for viral replication and transcription are discussed.

INTRODUCTION

Papillomaviruses induce benign papillomas or warts in man (Rowson & Mahy, 1967) and in a variety of animals (Lancaster & Olson, 1982). The human papillomaviruses (HPVs) are an extremely diverse group comprising more than 30 distinct types (Gissmann & Schwartz, 1985); they contain a circular double-stranded DNA genome of approximately 8 kb (DeLap et al., 1977).

Evidence strongly suggests that certain types of HPV are closely associated with specific human cancers; for example, epidermal cancer in patients with epidermodysplasia verruciformis is associated with HPV types 5 and 8 (Lutzner et al., 1984). More recently, the proposal that HPVs are implicated in human genital cancer has been strengthened by detection of specific types (HPV-16 and HPV-18) in a high percentage of genital cancer biopsies (Dürst et al., 1983; Boshart et al., 1984; Scholl et al., 1985; Di Luca et al., 1986; Macnab et al., 1986).

We have detected HPV-16 and HPV-18 DNA sequences in genital cancer biopsies from patients in the west of Scotland and north-east of England (Macnab et al., 1986). Here, we describe the molecular cloning of HPV-16 DNA sequences from one of these biopsies (a vulvar carcinoma in situ) and present evidence for a head-to-tail tandem arrangement of HPV-16 genomes. Our data provide evidence for HPV-16 dimers present as circular episomal elements in the tumour DNA. We demonstrate that these HPV-16 genomes contain a deletion within the non-coding region (NCR) located between the early and late transcribed regions.

METHODS

DNA, bacteriophage and bacterial stocks. The bacteriophage lambda vector EMBL3 and Escherichia coli strains K803, NM538 and NM539 (Frischauf et al., 1983) were kindly provided by Dr D. P. Leader. Human
papillomavirus prototype genomes HPV-6, HPV-11, HPV-16 and HPV-18, cloned into pBR322, were provided by Drs H. zur Hausen and L. Gissmann. For use as DNA probes, prototype HPV DNAs were cleaved with the appropriate restriction endonucleases and separated from pBR322 sequences by two consecutive rounds of electrophoresis through low melting point agarose.

**Isolation of genomic DNA from tumour biopsies.** Tumour biopsies were surgically removed by Dr S. Walkinshaw as previously described (Macnab et al., 1986); the tumour biopsies described here were isolated from Caucasian women indigenous to the west of Scotland. Tumour DNA was prepared from tissue confined to malignant or histologically abnormal regions. Genomic DNA was extracted from biopsy samples in 0·4 % SDS, 50 mm-Tris-HCl pH 8·0, 10 mm-EDTA, 0·1 M-NaCl containing proteinase K (200 μg/ml) and phenol-extracted (Varmus & Bishop, 1973), digested with RNase A (100 μg/ml) and re-extracted sequentially with phenol and chloroform. The DNA was dialysed extensively against 0·1 × SSC (1 × SSC is 0·15 M-NaCl, 15 mm-sodium citrate, pH 7), precipitated with ethanol and resuspended in H2O.

**Restriction endonuclease digestion and agarose gel electrophoresis.** DNA was digested with appropriate restriction endonucleases (obtained from Gibco/Bethesda Research Laboratories) under conditions recommended by the supplier. Samples were analysed by electrophoresis in horizontal agarose slab gels in a buffer containing 36 mm-Tris pH 7·8, 30 mm-NaH2PO4, 1 mm-EDTA. Following electrophoresis, DNA was transferred to nitrocellulose membranes (Schleicher & Schuell BA85) or Gene Screen Plus membranes (New England Nuclear) as described by Southern (1977) and hybridized with radiolabelled HPV-16 DNA as outlined below or under conditions previously described (Macnab et al., 1986).

**Construction and screening of genomic DNA library.** Genomic DNA was partially digested with Sau3AI, treated with phosphatase and ligated with the lambdα vector EMBL3 which had previously been digested with BamHI and EcoRI essentially as described by Frischauf et al. (1983). The in vitro packaging, phage amplification and propagation steps were carried out using standard procedures (Sternberg et al., 1977; Maniatis et al., 1982). A library of 106 recombinant phage (approximately 1 genome equivalent of cellular DNA) was screened for HPV-16 DNA sequences and positive phage were isolated by standard procedures (Benton & Davis, 1977; Maniatis et al., 1982). For hybridizations, cloned HPV DNA was cleaved and separated from pBR322 sequences as described above, prior to labelling with [α-32P]dATP and [α-32P]dTTTP by nick translation to a specific activity of approximately 106 c.p.m./μg (Rigby et al., 1977). The filter membranes were prehybridized at 65 °C in 5 × SSPE (1 × SSPE is 0·18 M-NaCl, 10 mm-NaH2PO4, pH 7·4, 1 mm-EDTA), 5 × Denhardt's solution (1 × is 0·02 % Ficoll 400, 0·02 % polyvinylpyrrolidone, 0·02 % bovine serum albumin), 0·1 % SDS and 100 μg/ml denatured calf thymus DNA. The hybridization mixture contained radiolabelled HPV-16 DNA (106 c.p.m./ml hybridization solution, 0·1 ml hybridization solution/cm² filter area). After incubation at 65 °C for 1 to 3 days, the filters were washed three or four times for 10 min in 2 × SSC, 0·1 % SDS followed by two washes in 1 × SSC, 0·1 % SDS at 65 °C for 1 to 2 h, air-dried and exposed to Kodak XAR-5 films. In digests involving DNA from phage clones, insert sequences were released from vector sequences by digestion with SalI before further digestion with other restriction endonucleases. The SalI site is located immediately adjacent to the Sau3A1/BamHI site on the vector polylinker sequence (Frischauf et al., 1983).

**RESULTS**

Genomic DNA was isolated from a variety of genital tumour biopsies and samples (20 μg) were digested with endonuclease BamHI which cleaves HPV-16 DNA at a unique site (Dürst et al., 1983), subjected to agarose gel electrophoresis, transferred to Gene Screen Plus membranes and hybridized with radiolabelled HPV-16 DNA. The copy number of HPV-16 DNA in the tumour DNAs was estimated by comparing the tumour DNA hybridization to that obtained with known standard amounts of HPV-16 DNA (Fig. 1, lanes 1 and 2). The vulvar carcinoma (Vu27; Fig. 1, lane 3) contained several different HPV-16-hybridizing bands whereas the cervical carcinoma (CaCx23; Fig. 1, lane 4) contained a single hybridizing band of approximately 8 kb at the position of linearized HPV-16 genomic DNA. Both of these tumours contained HPV-16 DNA sequences at a level of three to five genome equivalents/cell. The cervical carcinoma (CaCx1; Fig. 1, lane 6) was HPV-16 DNA-negative at the level of one genome equivalent/cell, whereas the vulvar carcinoma in situ (Vu28; Fig. 1, lane 5) contained several hybridizing bands and HPV-16 DNA was present at approximately 100 genome equivalents/cell. The multiple bands of HPV-16 DNA (Fig. 1, lanes 3 and 5) may have been the result of incomplete digestion with BamHI or various species of HPV-16 DNA may have been present in the tumour DNA. HPV-18 DNA was shown to hybridize to Vu28 tumour DNA although the PsI digest of the DNA closely resembled an HPV-16 genome cleavage pattern (Macnab et al., 1986). Further, when Vu28 was cleaved with BamHI no hybridizing band of 1 kb
characteristic of an HPV-18 genome was detected. The data suggest that some cross-homology, not yet exactly identified, was present between the prototype HPV-18 DNA and certain HPV-16-containing tumour DNA isolates. Under the hybridization conditions used here, there appeared to be no homology between the prototype HPV-16 and the prototype HPV-18 DNA probes (Fig. 2, lane 5).

As the DNA from tumour Vu28 contained a high copy number of HPV-16 genomes, we decided to analyse the arrangement of HPV-16 DNA sequences in this tumour. Thus, a genomic DNA library was constructed by inserting DNA from tumour Vu28, partially digested with Sau3AI, into the bacteriophage lambda vector EMBL3 which was then amplified. Recombinant phages containing HPV-16 sequences were identified by plaque hybridizations using a radiolabelled HPV-16 DNA probe.

Five recombinant phage clones were isolated and their DNAs analysed by restriction endonuclease digestion and blot hybridization. Results for all five recombinants were essentially the same; indeed, three clones appeared identical and may have been siblings derived from the amplification step. Therefore, analysis of a single representative clone (λHPV-16.1) is presented to illustrate the structure. A summary of the restriction maps of all the clones is included.
Fig. 2. Comparison of the hybridization pattern of phage clone λHPV-16.1 with relevant papillomavirus DNAs. DNA from phage clone λHPV-16.1 was digested with BamHI and PstI (lane 1) and hybridization to a radiolabelled HPV-16 DNA probe was compared with BamHI/PstI digests of HPV-16 (lane 2), of HPV-6 (lane 3), of HPV-11 (lane 4), and an EcoRI/PstI digest of HPV-18 (lane 5). M, λ HindIII size markers in kb; letters a to e, hybridizing fragments of HPV-16 DNA (PstI f and g are small fragments which under these conditions of electrophoresis ran off the gel).

Restriction endonuclease digestions were performed to confirm that the clones contained HPV-16 DNA sequences, because the library was screened under relatively non-stringent conditions designed to optimize the detection of clones and it was possible that positive clones contained papillomavirus DNA sequences related to but not identical with HPV-16. Thus, HPV-16, HPV-11 and HPV-6 DNAs were digested with BamHI and PstI and HPV-18 DNA was digested with EcoRI and PstI. Digests were compared with BamHI/PstI-digested DNA from clone λHPV-16.1. The results (Fig. 2) showed hybridization of these DNAs to a
Fig. 3. Comparison of hybridization pattern obtained with clone HPV-16.1 with that obtained with HPV-16 DNA following digestion with the relevant restriction endonucleases. The hybridization probe was radiolabelled HPV-16 DNA. Restriction endonuclease digests were: lane 1, λHPV-16.1 BamHI; lane 2, λHPV-16.1 BamHI plus three HPV-16 non-cutting enzymes (XhoI, SstI, HindIII); lane 3, λHPV-16.1 BamHI plus TaqI; lane 4, λHPV-16.1 TaqI; lane 5, HPV-16 BamHI plus TaqI; lane 6, λHPV-16.1 BamHI plus HincII; lane 7, λHPV-16.1 HincII; lane 8, HPV-16 BamHI plus HincII. M, λ HindIII size markers in kb.

radiolabelled HPV-16 DNA probe. The probe hybridized strongly to λHPV-16.1 (Fig. 2, lane 1) with no hybridization detected to HPV-6, HPV-11 or HPV-18 (lanes 3, 4 and 5 respectively); further, the cleavage pattern of λHPV-16.1 (lane 1) closely resembled that of HPV-16 (lane 2), except that PstI b in λHPV-16.1 was slightly smaller. This analysis indicated that clone λHPV-16.1 contained HPV-16 DNA sequences.

The clones, each of which contained approximately 16 kb of insert DNA, were further analysed to determine whether cellular DNA sequences were present in addition to the HPV-16 sequences. This was done by digesting the cloned DNAs with BamHI, which cleaves HPV-16 DNA at a single site, and then comparing the hybridization pattern with patterns generated by digestion with BamHI in combination with three HPV-16 non-cutting restriction endonucleases (XhoI, SstI and HindIII). Under these conditions, any cellular DNA sequences present would be
expected to contain cleavage sites for at least one of the three additional enzymes, thus resulting in HPV-16-containing fragments with altered electrophoretic mobility. Results for clone λHPV-16.1 (Fig. 3: compare lanes 1 and 2) show that the same three bands (sizes 7.9 kb, 6.9 kb and 1.05 kb) were present in single and multiple digests indicating that all three bands contained HPV-16 sequences and strongly suggesting that no cellular DNA was present. In addition, the sizes of the λHPV-16.1 BamHI DNA fragments added up to approximately 16 kb, the size of the insert which could be detected as undigested DNA in Fig. 3, lanes 1 and 2.

The structures of the DNA clones were further analysed by digestion with other HPV-16 single cut restriction endonuclease (TaqI and HincII) either singly or in a double digest with BamHI and then comparing the hybridization patterns with the appropriate double digests generated with HPV-16 prototype DNA (Fig. 3, lanes 3 to 8). Clone λHPV-16.1 digested with TaqI produced three bands of 7.9 kb, 4.6 kb and 3.35 kb that hybridized to HPV-16 (Fig. 3, lane...
Fig. 5. Localization of the deletion in phage clone λHPV-16.1 to an HPV-16 DNA fragment spanning the NCR.DNAs from clone λHPV-16.1 or HPV-16 were digested with various restriction endonucleases and the hybridization patterns (obtained with a radiolabelled HPV-16 probe) were compared. Restriction endonuclease digests were: lane 1, λHPV-16.1 BamHI plus PstI; lane 2, HPV-16 BamHI plus PstI; lane 3, λHPV-16.1 BamHI plus AvaII; lane 4, HPV16 BamHI plus AvaII; lane 5, λHPV-16.1 BamHI plus TaqI; lane 6, HPV-16 BamHI plus TaqI. Letters a to g, hybridizing fragments of HPV-16 DNA; M, λ HindIII size markers in kb.

4) and three bands of 7.9 kb, 6.1 kb and 1.95 kb with HincII (Fig. 3, lane 7). The 7.9 kb band, observed with all three single cut enzymes, was likely to have been full-length HPV-16 DNA and hence the two smaller bands represented flanking partial copies of the HPV-16 genome. This structure was confirmed by the double digests (Fig. 3, lanes 3 and 6) which produced only
four bands of which two (5.6 kb and 2.3 kb with TaqI/BamHI; 5.0 kb and 3.0 kb with HincII/BamHI) corresponded to similar bands present in the appropriate double digests of HPV-16 genomic DNA; each of the other two bands was present in the appropriate single digests.

As the BamHI and HincII sites on the HPV-16 genome are 5 kb apart (Fig. 4b; Seedorf et al., 1985), there must be at least 13 kb (approximately 1.6 genome equivalents) of HPV-16 DNA present in a head-to-tail orientation to produce full-length HPV-16 DNA when digested with these enzymes. Thus, it is probable that all 16 kb of the λHPV-16.1 insert DNA (two genome equivalents) was HPV-16 DNA arranged in a head-to-tail configuration. From the mapping data (and similar data for the other HPV-16-containing clones) we were able to construct detailed restriction maps for all five clones (Fig. 4a). These maps are consistent with additional data obtained by digestion of the clones with other enzymes for which the cleavage sites are shown in Fig. 4(b).

As noted earlier (Fig. 2: compare lanes 1 and 2) PstI b of clone λHPV-16.1 was smaller than the PstI b of HPV-16 prototype DNA. All the five clones isolated had a similar relative deletion within PstI b and this had occurred in each HPV-16 copy of the cloned dimer. The position of this deletion was further localized by comparing BamHI/AvaII and BamHI/TaqI double digests of clone λHPV-16.1 with similar double digests of HPV-16 genomic DNA (Fig. 5). The deletion was within the overlapping fragments PstI b, AvaII c and BamHI/TaqI b. With reference to the restriction map (Fig. 4b) and nucleotide sequence of HPV-16 (Seedorf et al., 1985) the deletion was localized to a 1 kb region (shown in Fig. 4b) which spans the NCR of the HPV-16 genome. This NCR contains the origin of DNA replication and transcriptional control sequences. The PstI b fragment from clone λHPV-16.1 has been subcloned into a pUC13 plasmid vector. Fine mapping and sequencing data (unpublished observations) indicate that the deletion extends for 121 bp from the G residue at position 7277 (or T residue at position 7278) to the T residue at position 7400 (or G residue at position 7401) in the numbering system according to Seedorf et al. (1985). This confirms that the deletion is localized within the NCR and does not extend into the 3' end of the L1 open reading frame.

**DISCUSSION**

In the present study, we have shown that HPV-16 DNA sequences isolated from a vulvar carcinoma in situ are present in a head-to-tail tandem arrangement. As the sizes of the cloned inserts are approximately 16 kb and the size of HPV-16 prototype DNA is 7904 bp (Seedorf et al., 1985) this implies that there may be exactly two genomes of HPV-16 present in the clones. Moreover, for each clone the Sau3AI sites located at both ends of the cloned inserts appear to be identical and the three non-identical clones show selection of a different Sau3AI cloning site (Fig. 4a). Inserts having this structure are unlikely to have been produced from an integrated tandem array of HPV-16 genomes as this would require two Sau3AI cuts at identical positions on two HPV-16 genomes separated by 16 kb (e.g. in genomes I and III Fig. 6b). The probability of this occurring depends on the number of other Sau3AI sites present within 15 to 20 kb of the first Sau3AI cut (this length of 15 to 20 kb is the average size distribution of the tumour DNA used in cloning following partial digestion with Sau3AI). For the λHPV-16.1 insert the number of such Sau3AI sites is 10 and the probability is therefore 1/10 or 0.1. For the inserts λHPV-16.2, 3, 4 and 5 the number of Sau3AI sites is eight and the probability is 1/8 or 0-125. The combined probability of all three inserts being 16 kb with identical ends is the product of the individual probabilities i.e. 1/640 or 0.00156. A much more likely event resulting in the generation of the cloned inserts is a single Sau3AI cleavage of a dimeric episomal arrangement of HPV-16 genomes (Fig. 6a). Such a cleavage at any one of the 22 available Sau3AI sites in this arrangement will always produce inserts of 16 kb with identical ends, with a resulting probability of 1/0. For each insert arrangement there is a choice of two possible cleavage sites in the dimer (see Fig. 6a).

It is well established that papillomavirus genomes are present in transformed and tumour cells as autonomously replicating episomal structures (Amtmann et al., 1980; Law et al., 1981;
Fig. 6. Possible structures from which the cloned HPV-16 DNA inserts may be derived. Small arrows indicate all Sau3AI cleavage sites and large arrows indicate the Sau3AI cleavage sites used during the cloning step. The unique HPV-16 BamHI site is indicated. (B). (a) Dimeric circular structure. (b) Linear array of HPV-16 genomes labelled I, II and III. In both structures, HPV-16 genomes are in the head-to-tail arrangement.

Lancaster, 1981; Moar et al., 1981; Campo & Spandidos, 1983; Groff et al., 1983). In these cases, the papillomavirus genomes generally were present as episomal monomers although Law et al. (1981), Lancaster (1981) and Groff et al. (1983) described a fraction which may represent concatemeric forms. Binetruy et al. (1982) transformed rodent cells with bovine papillomavirus type 1 (BPV-1) linked to plasmids pBR322 or pML2; both virus and plasmid sequences were maintained as non-integrated molecules, predominantly as oligomeric forms in head-to-tail arrangement. Further, Waldeck et al. (1984) found by electron microscopy that BPV-1 genomes in transformed hamster cells were present as monomeric and dimeric episomes at nearly equal frequency. In studies of other circular DNA molecules which replicate autonomously such as mitochondrial DNA (Hudson & Vinograd, 1969), polyoma virus (Cuzin et al., 1970) and simian virus 40 (SV40) (Jaenisch & Levine, 1971), oligomeric circular molecules have been observed; the oligomeric SV40 DNA was infectious.

In recent work with HPVs (Dürst et al., 1985; Boshart et al., 1984) HPV-16 and HPV-18 DNAs have been detected in genital cancer biopsies as oligomeric episomes (analogous to the structures detected here), as monomeric episomes and as sequences integrated into the host genome. The evidence for oligomeric episomes was obtained from CsCl centrifugation or two-dimensional gel electrophoresis. Here, we present direct evidence for the existence of oligomeric molecules in human genital tumours by molecular cloning of putative dimeric episomal structures from tumour DNA.

Although only dimeric HPV-16 genomes were isolated in this study, the presence in the tumour DNA of integrated sequences, and/or monomeric or higher oligomeric extrachromosomal structures is not excluded. Indeed, these structures are less likely to be observed here due to the partial Sau3AI cleavage employed during cloning and to the insert size optimum (10 to 20 kb) of the cloning vector.

The observation that the HPV-16 molecules characterized contain deletions is not particularly unusual. Deletions in papillomavirus isolates have been observed by others (Amtmann et al.,
1980; Ostow et al., 1982; Binetruy et al., 1982; Schwartz et al., 1985). However, these deletions were mainly in the late region which is not usually transcribed in transformed and tumour cells (Amtmann & Sauer, 1982; Heilman et al., 1982) and none have been described within the NCR which contains the origin of viral DNA replication and early gene transcriptional control signals.

HPV-6 isolates have been obtained from an invasive vulvar carcinoma (Rando et al., 1986) and a Buschke-Löwenstein tumour (Boshart & zur Hausen, 1986) which contain viral NCR sequence duplications. In one case (Rando et al., 1986), there was also evidence of insertions within the NCR of DNA with homology to cellular sequences. The nucleotide sequence for HPV-33 (Cole & Streeck, 1986), isolated from a cervical carcinoma (Beaudenon et al., 1986) also reveals tandem repeats of NCR sequences; these observations may be consistent with viral sequence duplications within the SV40 and polyoma virus enhancer and Moloney murine sarcoma virus long terminal repeat regions (Benoist & Chambon, 1981; Ruley & Fried, 1983; Dhar et al., 1980). Studies with the human BK papovavirus (BKV) have shown that a plaque morphology mutant contains deletions and rearrangements of DNA within the viral NCR, near the origin of replication, and this virus exhibited increased transforming ability as compared to wild-type BKV (Watanabe & Yoshike, 1982; Watanabe et al., 1984a). The increased transforming ability of the mutant has been ascribed to its capacity to express early viral functions constitutively (Watanabe et al., 1984b).

Alterations within the HPV-16 NCR could affect the ability to replicate and/or express viral genes in tumour cells. The virus that originally infected the cells may have contained the deletion or the deletion may have been generated in the course of viral DNA persistence in the cells. The deletion is unlikely to be responsible for the production of oligomeric molecules since these appear to be a product of the replication of circular genomes which do not have alterations within the NCR. One possibility is that the deletion may affect the frequency of molecules in the tumour cells; the HPV-16 copy number was greater than 100. There is no direct evidence to suggest that the dimeric molecules are replication-competent, although as we have obtained several clones this may suggest a relatively high abundance.

We thank Professor J. H. Subak-Sharpe for his continuing interest, Dr S. Walkinshaw for providing tumour biopsies and Dr R. Thompson for reading the manuscript. Thanks to Ms F. Conway and Mrs J. Mavor for secretarial assistance. This work was supported by Cancer Research Campaign grant SP1341.

REFERENCES

BINETRUY, B., MENEGUZZI, G., BREATHNACH, R. & CUZIN, F. (1982). Recombinant DNA molecules comprising bovine papillomavirus type 1 DNA linked to plasmid DNA are maintained in a plasmidial state both in rodent fibroblasts and in bacterial cells. EMBO Journal 1, 621-628.
HPV-16 dimeric episomes in vulvar carcinoma


(Received 30 July 1986)